Effect of cadmium on cytosine hydroxymethylation in gastropod hepatopancreas

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Abstract

5-hydroxymethylcytosine (5hmC) is an important, yet poorly understood epigenetic DNA modification, especially in invertebrates. Aberrant genome-wide 5hmC levels have been associated with cadmium (Cd) exposure in humans, but such information is sorely lacking for invertebrate bioindicators. Here, we aimed to determine whether this epigenetic mark is present in DNA of the hepatopancreas of the land snail *Cantareus aspersus* and is responsive to Cd exposure. Adult snails were reared under laboratory conditions and exposed to graded amounts of dietary cadmium for 14 days. Weight gain was used as a sublethal endpoint, whereas survival as a lethal endpoint. Our results are the first to provide evidence for the presence of 5hmC in DNA of terrestrial mollusks; 5hmC levels are generally low with the measured values falling below 0.03%. This is also the first study to investigate the interplay of Cd with DNA hydroxymethylation levels in a non-human animal study system. Cadmium retention in the hepatopancreas of *C. aspersus* increased from a dietary Cd dose of 1 milligram per kilogram dry weight (mg/kg d. wt). For the same treatment, we identified the only significant elevation in percentage of samples with detectable 5hmC levels despite the lack of significant mortalities and changes in weight gain among treatment groups. These findings indicate that 5hmC epigenetic mark may be responsive to Cd exposure, thereby opening a new aspect to invertebrate environmental epigenetics.

Short title: Dietary Cadmium and Gastropod DNA hydroxymethylation

Keywords: cadmium, 5-hydroxymethylcytosine, land snails, hepatopancreas, dietary exposure

1. Introduction

Methylation of DNA at the 5 position of cytosine (5-methylcytosine or 5mC) is pivotal to DNA metabolism and gene expression in most eukaryotes (Jones 2012), including invertebrates (Kucharski et al. 2008; Lyko et al. 2010; Riviere et al. 2013; Lian et al. 2015). The oxidation product of 5mC, 5-hydroxymethylcytosine (5hmC) is also an important, yet poorly understood epigenetic mark, which in vertebrates appears to be linked to cytosine demethylation events and transcriptionally active genomic loci (Branco et al. 2011; Bergman and Cedar 2013). To date, the presence of 5hmC has been confirmed in certain invertebrate phyla, such as arthropods (Cingolani et al. 2013; Felicielo et al. 2013; Wojciechowski et al. 2014; Strepetkaitė et al. 2016) or ctenophores (Moroz et al. 2014). However, we know amazingly little about the occurrence of this cytosine base modification in mollusks – the second largest phylum in the animal kingdom – despite their ecological importance and valuable role as bioindicators of environmental health (Dallinger et al. 2001; Oehlmann and Schulte-Oehlmann 2003).

A ubiquitous element within the environment, cadmium (Cd) is considered a persistent, bioaccumulative priority contaminant (Spurgeon et al. 2008; ATSDR 2012), and therefore, has attracted considerable research interest from environmental scientists. The main route of Cd uptake for terrestrial animals is via food, with the renal and hepatic tissues serving as the primary retention endpoints. Once accumulated, this trace metal interferes with multiple epigenetic mechanisms, including DNA methylation (Wang et al. 2012). Evidence of the potential for cadmium to affect the DNA methylation cycle originates from studies with 5mC (Takiguchi et al. 2003; Jiang et al. 2008; Hanna et al. 2012; Hossain et al. 2012; Pierron et al. 2013; Zhang et al. 2013; Sanders et al. 2014), but only rarely with 5hmC (Tellez-Plaza et al. 2014). Moreover, the interplay between Cd and 5hmC in invertebrate ecotoxicological models has not been investigated before.

Land snails (*Pulmonata*) are suitable study systems for cadmium hazard. First, these synanthropic invertebrates are effective cadmium bioacumulators and tolerate tissue concentrations far above environmental levels without showing any major metabolic side effects (Dallinger et al. 2001). Second, commonly used animal models in toxicological studies (e.g., rats, mice, fish) have separate sexes, both of which have to be tested for providing relevant results on epigenetic effects of anthropogenic pollutants. Land snails, by contrast, are hermaphrodites, and therefore, their use is a more cost-effective alternative to vertebrate study systems in epigenetic ecotoxicology. Third, the gastropod hepatopancreas fulfills the functions of both the mammalian liver and pancreas and functions as the main site for cadmium storage and detoxification. As a result, it is the most thouroughly studied target tissue related to Cd accumulation in this group of animals (e.g., Rabitsch 1996; Dallinger et al. 2004; Gimbert et al. 2008; Hispard et al. 2008; Hödl et al. 2010; Höckner et al. 2011; Nica et al. 2015).

We recently showed that the genomic DNA from the hepatopancreas of the Brown garden snail, *Cantareus aspersus* (syn. *Cornu aspersum* or *Helix aspersa*), does contain 5mC, an epigenetic mark that is responsive to cadmium (Nica et al. 2016). 5hmC – the epigenetic mark measured in this study – is a enzyme mediated oxidation product of 5mC and also appears to be responsive to environmental stressors (Dao et al. 2014; Zhang et al. 2014). For this reason, we addressed two key questions: (1) Does the DNA of the gastropod hepatopancreas contain 5hmC? (2) What is the relevance of the 5hmC content of hepatopancreas DNA as an endpoint of Cd exposure? In other words, is 5hmC responsive to oral intake of cadmium and could this epigenetic mark be used as a potential biomarker.

To test these hypotheses, adult specimens of *C aspersus* were exposed under laboratory conditions to graded, field-relevant amounts of dietary cadmium for 14 days. The topic of this work joins an emerging field of research using epigenetic approaches to address environmental problems – one of the next frontiers in the study of epigenetics (Head et al. 2012). Understanding this response is important for the study of pollution and biomonitoring the effect of heavy metals with invertebrates since it will provide a new perspective on the effects of cadmium on the DNA modification dynamics in these organisms and may help develop novel endpoints for cadmium exposure.

2. Materials and Methods

2.1. Preparation of the food

An artificial food was prepared by mixing 50 g fortified infant cereals (Nestle Nestum 5 – FiveCereals), 20 g carrot baby food (HiPP, UK), and 15 g agar (A-1296, Sigma) with double distilled water to obtain 1000 milliliters (mL) agar medium. A fungicide (1% methylparaben solution) was added to the solution as 3 mL per 1000 mL fodder. Each liter of medium was divided equally among forty Petri plates (about 25 mL/plate). After cooling, the Petri plates were maintained in a refrigerator for maximum one week. The spiked food was prepared similarly, using cadmium solutions of known concentrations, i.e., 0.02, 0.05, 0.2, 1, and 10 milligrams per liter (mg/L), instead of double distilled water. These concentrations were chosen based on the reference values for maximum Cd concentrations (expressed as milligrams per kilogram dry weight, mg/kg d. wt) allowed in vegetal foods on which the snails routinely feed, such as fruits (0.05 mg/kg d. wt), stem vegetables (0.1 mg/kg d. wt), and leafy vegetables (0.2 mg/kg d. wt) (MWFEP 2002; EC Regulation no.1881/2006); and Cd effects on cell proliferation, histopathological alterations, and DNA integrity in the snail hepatopancreas (Hödl et al. 2010; Itziou and Dimitriadis 2011). Cadmium chloride ($CdCl_2$, 99.99% trace metal basis, Sigma-Aldrich) was used as a source of Cd (Itziou et al. 2011; Nica et al. 2015). The treatments were abbreviated as 0Cd (controls), 0.02Cd, 0.05Cd, etc.

2.2. Snail feeding experiment

Newly matured *C. aspersus* snails (32 specimens, aged 12 months) obtained from the "Mokry Dwór" farm (Krzymów, Wielkopolska, Poland) were bred under controlled environmental conditions at the Laboratory Animal Facility of "Victor Babes" University of Medicine and Pharmacy from Timisoara (UMFT). This species is routinely used as a gastropod model in ecotoxicological studies because it has a well known physiology and is easily reared both under field and laboratory conditions (Garcia et al. 2006). The snails were of similar size (mean shell height 25.9 ± 1.34 mm) and had a thickened 'lip' at the mouth of their shells, showing adulthood (Kerney and Cameron 1979). Four snails were used for the 1Cd treatment, five for the 0Cd and 0.02Cd treatments, and six for the 0.05Cd, 0.2Cd, and 10Cd treatments, respectively. After transfer to a climate-controlled room (18-20°C, 12-h light : 12-h dark cycle), each animal was kept separately in a 600-mL aerated polypropylene terrarium with a perforated lid and maintained without food for 4 days to accommodate to laboratory conditions (Itziou et al. 2011).

After acclimatization, all test animals were maintained on Cd-spiked diets for 14 days. A layer of ash-free filter paper was placed on the bottom of each terrarium and wetted two times a day with double distilled water by using a pressure sprayer to provide a moist micro-environment for snail breeding. The daily activity schedule

involved fresh fodder supply, monitoring snail fitness, removal of uneaten food and faeces, and replacing the sheet of ash-free filter paper. The terrariums were cleaned three times a week with double distilled water.

For each specimen, the body weight was determined to the nearest 0.01 mg with an analytical balance (TP-214, Denver Instrument GmbH) at the start and the end of the experiment. Weight gain was calculated as the difference between these weights. This parameter is commonly used in ecotoxicological studies with gastropods as a sublethal endpoint of Cd exposure (e.g., Russell et al. 1981; Gomot 1997; Coeurdassier et al. 2002). Mortalities during the period of exposure were also recorded to quantify the lethal effects of Cd at the dietary doses used in the present experiment.

All surviving animals were collected at the end of the experiment and then sacrificed. The soft body was removed from the shell using a hemostat and the hepatopancreas was detached from the visceral mass. Hepatopancreas samples were collected, washed in double distilled water, dried on cellulose tissue, and stored at -80°C until further analyses. Both chemical and methylation analyses were conducted for all hepatopancreas samples collected.

2.3. Cadmium analyses

The frozen hepatopancreas samples were thawed, oven dried (105°C, 24 hours), and weighed to the nearest 0.01 mg using an analytical balance (TP-214, Denver Instrument GmbH). After calcination in a muffle furnace (Nabertherm B150, Lilienthal; 550°C, 6 hours), the ash was submitted to wet acid digestion. Briefly, the ash was treated with 0.5 mL of 65% HNO_3 (Merck, suprapure), heated to dryness, dissolved in 20 mL of 0.5 N HNO_3, and then filtered through ash-free filter paper. Finally, the volume of each sample was brought to 30 mL with 10 mL HNO_3 0.5 N.

Cadmium content in the filtrates was measured by flame (air-acetylene) atomic absorption spectrophotometry (VARIAN AA240FS) fitted with a metal-specific hollow-cathode lamp as a source of radiation. Mix standard solutions (1000 mg/L) of ICP Multielement Standard solution IV CertiPUR were purchased from Merck (Merck KGaA, Darmstadt, Germany). The reagents and standard solutions were prepared using double distilled water (spectroscopic pure). All glassware was treated with Pierce solution 20% (v/v), rinsed with cold tap water followed by washing 20% (v/v) nitric acid and rinsing them again with double-distilled water. Blanks and triplicate samples were also analyzed during the procedure to deliver homogeneous and accurate results. The Certified Reference Material (CRM) NCS DC 85105a (NCS Testing Technology Co., Ltd.; China Iron & Steel Research Institute Group, formerly Central Iron & Steel Research Institute) was used to verify the accuracy of acid-extraction of cadmium from hepatopancreas samples. The certified concentration value of cadmium in this standard reference material is 14 mg/kg (data available in the 2016-2017 catalog of NCS Reference Materials, http://www.ncsstandard.com/catalog.aspx). The percent recovery mean for acid-extraction of cadmium was 96%. The variation coefficients were below 8%, whereas the Cd detection limit, as determined by using the calibration curve method, was 0.01 mg/kg d. wt. The blank reagent and standard reference animal sample were included into each sample batch to verify the accuracy and precision of the digestion procedure, as well as for the subsequent analyses.

159 *2.4. DNA extraction and quantification analysis*

160 Total DNA was extracted from the hepatopancreas with the DNeasy Blood & Tissue Kit (Qiagen, Cat no.
161 69506) using the animal tissue bench protocol as recommended by kit user manual ("Purification of Total DNA
162 from Animal Tissues" as found in DNeasy Blood & Tissue Handbook, Qiagen, Valencia, CA). Once extracted,
163 the DNA was verified for quality (260/280 nm, NanoDrop-2000, Thermo Fisher Scientific Inc., USA). Total
164 5hmC content in DNA of the hepatopancreas of *C. aspersus* adults was determined with the Quest 5-hmC^{TM}
165 DNA ELISA Kit (Zymo Research, Cat no. D5426) according to the manufacturer's instructions. This sandwich
166 ELISA-based system uses capture and detection antibodies to detect and quantify 5hmC levels in a wide range of
167 input DNA, including animal, plant, and microbial genomic DNA. It can run in a qualitative format, in which the
168 presence of 5hmC in DNA samples is detected by comparing their absorbance (optical density) to that of the
169 negative control (0% 5hmC); as well as in a quantitative format, which implies the comparison of sample
170 absorbance to a standard curve generated from solutions of 5hmC at known concentrations. The detection
171 threshold of this kit is 0.02% 5hmC per 100 nanograms (ng) input DNA according to the instruction manual.
172 Numerous studies attest high levels of 5hmC sensitivity in DNA samples for this ELISA kit, with detection
173 possible well below the 0.01% range (e.g., Tellez-Plaza et al. 2014; Murata et al. 2015; Pells et al. 2015; Pirola
174 et al. 2015).

175 In brief, 100 ng of denaturated DNA and anti-5hmC polyclonal antibodies (1 µg/mL) were added to a 96-
176 well plate and incubated for 1 hour at 37 ºC. The plates were washed three times and then incubated again with
177 0.2 µg/mL of the horseradish peroxidase (HRP)-conjugated anti-DNA antibody at 37 °C for 30 min. Finally,
178 developer solution (DS) was added into wells and the color was allowed to develop for 15 minutes at room
179 temperature (20 °C). The quantification of 5hmC content within each sample was conducted in duplicate. The
180 optical density (OD) was determined at 450 nanometers (nm) using a microplate Reader Stat Fax 4200
181 (Awareness Technology, USA). For absolute 5-hmC quantification, a standard curve was obtained by plotting
182 the various concentrations of the positive controls against the corresponding ODs. The standad curve obtained
183 was excellent (Fig. 1A), attesting to the reliability of 5hmC measurements.

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185 *2.5. Statistical analysis*

186 The inter-group homogeneity of body weight at the start of the experiment was tested with a Kruskall-Wallis
187 test with tied ranks. Differences in weight gain and hepatopancreas Cd content among treatments were analyzed
188 in the same manner, with post hoc Dunn's tests being applied in case of significant differences. A similar
189 approach was planned to be used for the percentage of 5hmC in hepatopancreas samples. If the measured values
190 were below those of the lowest positive control (0.03%), an qualitative ELISA approach was used to achieve a
191 yes or no answer indicating whether detectable levels of 5hmC antigen were present in the samples. The limit of
192 detection value (LoD), also known as the cutt-off value, was calculated as average OD of negative control plus
193 two standard deviations (SD) (Wattanaphansak et al. 2008). ODs above this threshold were considered to
194 indicate a detectable 5hmC signal ("yes" response). Data quality was assessed based on the value of the
195 coefficient of determination (R^2) corresponding to the ELISA standard curve obtained; and that of the intra-assay
196 coefficient of variation (C.V.), which should fell below 10% (HHS 2001). Pairwise comparisons with Fisher's
197 exact tests (one-tailed) were applied on percentages of yes/no responses for controls (as a benchmark group) and
198 those measured for different treatments (10Cd vs. 0Cd, 1Cd vs. 0Cd, etc.). Finally, an univariate logistic

regression was conducted to determine the relationship between the hepatopancreas cadmium concentrations (as a predictor variable) and the corresponding 5hmC levels (as a criterion variable). A p value < 0.05 was considered significant.

3. Results and Discussions

At the start of the experiment, different snail groups showed similar body weights (p = 0.423, Table 1), confirming the homogeneity of our samples. At 14 days, there were no significant differences in body weight gain among treatment groups (p = 0.548, Table 1). Moreover, no deaths occurred during the study period. These data show that, under the present experimental conditions, dietary Cd doses up to 10 mg/kg d. wt did not affect key organism-level toxicity endpoints, such as survival and weight gain. Identical results were obtained in a previous study using similar experimental conditions (Russel et al. 1981). These data attest to the increased tolerance of this species to cadmium exposure.

Table 1. Initial body weight and weight gain at the end of the experiment for the six treatments.

Treatment	N	Initial weight		Weight gain	
0Cd	5	8.35	(0.71)	1.36	(0.16)
0.02Cd	5	8.29	(1.14)	1.27	(0.46)
0.05Cd	6	8.97	(1.16)	1.12	(0.35)
0.2Cd	6	9.10	(0.93)	1.25	(0.33)
1Cd	4	9.09	(0.90)	1.43	(0.19)
10Cd	6	9.69	(1.64)	1.35	(0.56)

Initial body weight was measured at the start of the experiment, whereas the weight gain was calculated as the difference between the body weight at the end of the experiment (14 days) and that determined at the start of the experiment (0 days).

The gastropod hepatopancreas is an excellent bioaccumulator of environmental cadmium (Dallinger and Rainbow 1993), and hence a reliable tool for exposure measurement (Rabitsch 1996; Nica et al. 2015). Median concentrations measured here (Fig. 1A) ranged between 1.44 mg/kg d. wt (0.05Cd treatment) and 73.67 mg/kg d. wt (10Cd treatment). These values are largely comparable to those determined in mature specimens of *C. aspersus* and other gastropod species, such as *Eobania vermiculata* or *Cepaea hortensis*, when treated at similar doses (Russel et al. 1981; Laskowski and Hopkin 1996; Dallinger et al. 2004; Hispard et al. 2008; Itziou and Dimitriadis 2011).

We found significant differences in Cd content of the hepatopancreas among the six treatments (p < 0.001; Figure 1A). When compared to controls, the measured values were consistently elevated in the 1Cd snails (p = 0.045) and the 10Cd snails (p < 0.001), but no effect was observed for the three lowest treatments ($p \geq 0.137$). This trend is in line with the previous results (Laskowski and Hopkin 1996). Therefore, one can expect that this gastropod is able to maintain stable Cd levels in the hepatopancreas for low, but field-relevant dietary exposure. Cadmium excretion via mucus and/or faecal fluids, as well as its relocation into the foot (Gimbert et al. 2006; Notten et al. 2006), may account for these results.

A hormesis effect was observed at the two highest doses (Table 1). This suggests that mature snails, *C. aspersus*, may exhibit higher fitness compared to controls following a 14-day dietary exposure to slightly elevated Cd concentrations. This response was previously described in younger specimens (five- and eight-

week-old juveniles) after ingestion of a lightly contaminated diet (50 mg/kg d. wt Cd) or a moderately contaminated substrate (100 mg/kg d. wt Cd) for up to one month (Gomot 1997; Coeudassier et al. 2002). The internal Cd range for which hormesis occurred was between 0 and 40 mg/kg d. wt (Coeudassier et al. 2002), but the level to which this trace metal was retained in the hepatopancreas was not measured. No pertinent conclusions about the potential interplay between age and internal cadmium concentrations in shaping this response can be derived from these data. However, adult weight gain does not appear to be a reliable ecotoxicological endpoint for Cd exposure, at least for a short-term exposure to environmentally relevant dietary cadmiu concentrations. Our findings differ from those reported by Russel et al. (1981) who observed dose-dependent growth inhibition in mature specimens of similar size at higher dietary Cd doses (Russel et al. 1981)

Here, we provide the first evidence for the presence of 5hmC in land snails. Hence, these results expand our knowledge on DNA hydroxymethylation in gastropods, which until now was limited to data derived from two studies dealing with the aquatic gastropods *Biomphalaria glabrata* and *Aplysia californica* (Fneich et al. 2013; Moroz and Kohn 2013). It is also important to note that the presence of 5hmC in DNA of the molluskan hepatopancreas has not been reported before.

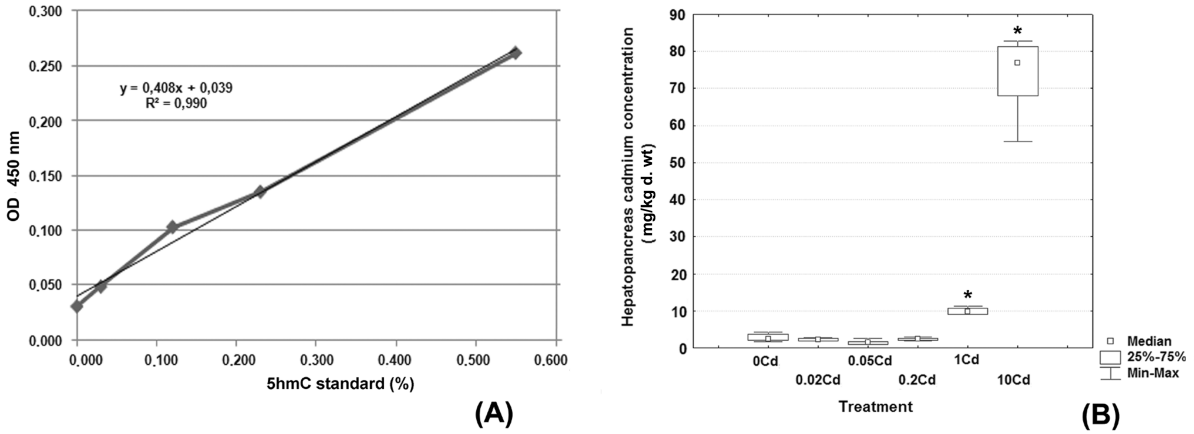

Fig 1 (A) ELISA standard curve. The upper left shows the standard curve equation and the corresponding coefficient of determination. (**B) Cadmium concentrations (mg kg^{-1} DW) in the hepatopancreas of *Cantareus aspersus* adults.** Marked boxes (*) indicate significant differences as compared to the reference group (Dunn's test, $p<0.05$).

Low, but detectable levels of 5hmC (< 0.03%) were observed in 9 out of 32 samples analyzed (28.12%; Fig. 2A). These values fell well below those measured in the human liver using a similar antibody-based approach (0.45% of total DNA, i.e., 0.09% of the total cytosine in the DNA; Li and Liu 2011) or in the murine liver using high-performance liquid chromatography-mass spectrometry (HPLC-MS) (0.05 to 0.07% from the total cytosine; Globisch et al. 2010). To our knowledge, such information is lacking for invertebrate organs with liver-like functions, including the molluskan hepatopancreas. Global 5hmC content of other tisues is, however, similarly low. For example, in the honey bee, *Apis mellifera*, the measured values by liquid chromatography-tandem mass spectrometry (LC-MS/MS) ranged between 0.0004% and 0.0006% of the total cytosine pool in the brain and abdomen, respectively (Rasmussen et al. 2016). With respect to gastropods, similar data originate from only one study, in which application of LC-MS/MS showed that only 0.0009% of total cytosines are hydroxymethylated in the genomic DNA from the soft tissue of the foot of *Biomphalaria glabrata* (Fneich et al.

2013). Despite methodological differences, this is quite consistent with our results. Taken toghether, these findings suggest that 5hmC is present in relatively small amounts in DNA from tested gastropod tissues.

This is also the first study to investigate the interplay of Cd with DNA hydroxymethylation in a non-human animal study system. No clear dose-response relationship was observed between cadmium concentration and total 5hmC content in the hepatopancreas of *C. aspersus* adults ($p = 0.748$). The percentage of samples with detectable 5hmC levels (Fig. 2B) was significantly higher compared to controls for the 1Cd treatment ($p = 0.039$), but not for the other treatments applied ($p \geq 0.575$). Hence, it appears that chronic 14-day exposure could modify global 5hmC levels in the hepatopancreas DNA of gastropods above a certain threshold level of dietary cadmium. From these findings, one may also expect that the corresponding dose-effect curve is not sigmoidal, but rather biphasic, displaying an inverted U- or J-shape. Indeed, such relationships have been reported in the toxicological literature, for methylation patterns in *Arabidopsis* plantlets that had been exposed to 0-5.0 mg/kg d. wt Cd for 15 days (Wang et al. 2016). The absence of a significant dose-response curve may also reflect an improper spacing of dose levels, which can hinder the characterization of this relationship (Hood 2016).

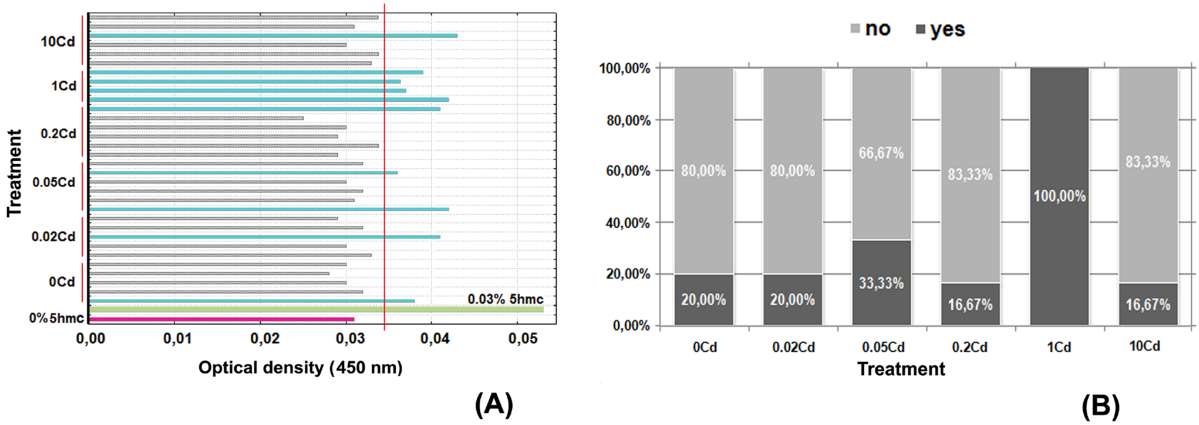

Fig 2 (A) Optical density (405-450 nm) for samples analyzed. Qualitative Elisa was used to achieve a yes or no answer indicating whether detectable levels of 5hmC antigens are present in samples. The vertical red line denotes the cut-off line. The purple column shows the OD of negative control, whereas the green column shows the OD of the lowest hydroxymethylated control, i.e., 0.03% 5hmC. Blue columns indicate a positive 5hmC signal, whereas grey columns denote a 5hmC signal below LoD.
(B) Percentage of yes/no responses for different treatments. Light grey denotes the percentage of samples with detectable levels of 5hmC antigens ("yes" response). Dark grey shows the percentage of samples with a 5hmC signal below the limit of detection ("no" response).

Interestingly, we identified the first consistent increase in cadmium retention in the hepatopancreas of *C. aspersus* adults at 1 mg/kg d. wt Cd in the diet. The same treatment yielded the single 5hmC peak observed in the present study. Given the putative role of this epigenetic mark in promoting gene expression during active demethylation (Branco et al. 2011; Hill et al. 2014), this association may suggest an increased likelihood of altered gene expression following low-level dietary cadmium exposure. It will be of interest to investigate the effects of cadmium on specific genes. In this context, the Cd-selective metallothionein (Cd-MT) gene deserves

particular consideration since it can be activated by dietary Cd levels as low as 2 mg/L (Baurand et al. 2015) and is generally inducible, unlike constitutively expressed genes with housekeeping functions (Hispard et al. 2008).

Overall, this preliminary study supports the presence of 5hmC in DNA of the hepatopancreas of *C. aspersus* and suggests that this methylation variant is, to a certain extent, responsive to cadmium exposure. For dietary Cd doses less 10 mg/kg d. wt in adult *C. aspersus* snails, this epigenetic mark seems to be more sensitive than traditional organismal endpoint measurements of Cd-related adverse effects, such as weight gain and survival. However, further investigations on this matter are needed before reaching a final conclusion. Thus, the present results should be confirmed using more precise and sensitive methods for measuring global DNA hydroxymethylation. LC-MS/MS can serve as an appropriate technique for achieving this purpose; it is considered the "gold standard" to accurately quantitate 5hmC levels in any DNA sample (Liu et al. 2013) and, as mentioned above, seems to have a limit of sensitivity less than a thousandths of a percent for gastropod DNA (Fneich et al. 2013). Such an approach will also help to avoid potential confounding factors in 5hmC quantification, e.g., cross-reactivity of 5hmC-antibodies with DNA methylated cytosines (Erdman et al. 2015). In addition, future studies should use a narrower spacing of exposure doses, using 1 mg/kg d. wt Cd in the diet as a benchmark dose for a 14-day continuous exposure. This could allow a more precise characterization of the dose-response relationship between cadmium concentrations and global 5hmC levels in the hepatopancreas of *C. aspersus* adults. Because of animal-to-animal variability in 5hmC content, larger sample sizes should also be used, thus increasing the statistical power of our study and the likelihood of obtaining significant results. However, such modest sample sizes are not unsual for pilot global DNA methylation studies, which was the case of our investigation.

4. Conclusions

5-hydroxymethylcytosine (5hmC) is an important, yet poorly understood epigenetic DNA modification, especially in mollusks. There is evidence for Cd effects on global DNA hydroxymethylation in humans, but not in invertebrate bioindicators. Here, we determined the changes induced in global 5hmC levels in DNA of the hepatopancreas of land snail *Cantareus aspersus* following a 14-day dietary exposure to low, but field-relevant Cd doses. Our results are the first to provide evidence for the presence of 5hmC in DNA of terrestrial mollusks and lend support that Cd may affect DNA hydroxymethylation in a non-human animal study system.

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References

ATSDR (2008) Toxicological profile for cadmium. US Department of Health and Human Services, Public Health Service, Atlanta, GA.

335 Baurand PE, Pedrini-Martha V, De Vaufleury A, Niederwanger M, et al (2015). Differential expression of
336 metallothionein isoforms in terrestrial snail embryos reflects early life stage adaptation to metal stress. PloS one
337 10: e0116004. doi: 10.1371/journal.pone.0116004.
338 Bergman Y, Cedar H (2013) DNA methylation dynamics in health and disease. Nat Struct Mol Biol 20: 274–
339 281. doi: 10.1038/nsmb.2518.
340 Branco MR, Ficz G, Reik W (2011) Uncovering the role of 5-hydroxymethylcytosine in the epigenome. Nat Rev
341 Genet 13: 7–13. doi:10.1038/nrg3080.
342 Cingolani P, Cao X, Khetani RS, Chen CC, Coon M, et al (2013) Intronic Non-CG DNA hydroxymethylation
343 and alternative mRNA splicing in honey bees. BMC Genomics 14: 666. doi: 10.1186/1471-2164-14-666.
344 Coeurdassier M, Gomot DVA, Lovy C, Badot PM (2002) Is the cadmium uptake from soil important in
345 bioaccumulation and toxic effects for snails? Ecotox Environ Saf 53(3): 425–431. doi: 10.1016/S0147-
346 6513(02)00004-0. pmid:12485588,
347 Dallinger R, Lagg B, Egg M, Schipflinger R, Chabicovsky M (2004) Cd accumulation and Cd-metallothionein
348 as a biomarker in *Cepaea hortensis* (Helicidae, Pulmonata) from laboratory exposure and metal-polluted
349 habitats. Ecotoxicology 13(8): 757–772. doi: 10.1007/s10646-003-4474-4. pmid:15736847.
350 Dallinger R, Berger B, Triebskorn-Kohler R, Kohler H (2001) Soil biology and ecotoxicology. In: Barker GM
351 (2001) The biology of terrestrial molluscs, CABI Publishing, Wallingford, pp. 489–525.
352 Dallinger R, Berger B, Hunziker PE, Birchler N, Hauer CR, Kägi JH (1993) Purification and primary structure
353 of snail metallothionein. Similarity of the N-terminal sequence with histones H4 and H2A. Eur J Biochem
354 216(3): 739–746.pmid:8404892.
355 Dallinger R, Rainbow PS (1993) Ecotoxicology of metals in invertebrates.Lewis Publishers, Boca Raton.
356 Dao T, Cheng RYS, Revelo MP, Mitzner W, Tang WY (2014) Hydroxymethylation as a novel environmental
357 biosensor. Environ Health Rep 1(1): 1–10. doi: 10.1007/s40572-013-0005-5.
358 Erdmann RM, Souza AL,Clish CB, Gehring M (2015) 5-Hydroxymethylcytosine is not present in appreciable
359 quantities in *Arabidopsis* DNA. G3: Genes Genomes Genetics 5(1): 1–8. doi: 10.1534/g3.114.014670.
360 European Comission (2006) Regulation No 1881/2006 of 19 December 2006 setting maximum levels for certain
361 contaminants in foodstuffs. Official Journal of the European Union L Series 364: 5–24.
362 Feliciello I, Parazajder J, Akrap I, Ugarkovic D (2013) First evidence of DNA methylation in insect *Tribolium*
363 *castaneum*. Environmental regulation of DNA methylation within heterochromatin. Epigenetics 8(5): 534–541.
364 doi: 10.4161/epi.24507.
365 Fneich S, Dheilly N, Adema C, Rognon A, Reichelt M et al (2013) 5-methyl-cytosine and 5-hydroxy-methyl-
366 cytosine in the genome of *Biomphalaria glabrata*, a snail intermediate host of *Schistosoma mansoni*. Parasite
367 Vector 6: 1. doi: 10.1186/1756-3305-6-167.
368 Garcia A, Perea JM, Mayoral A, Acero R, Martos J et al (2006) Laboratory rearing conditions for improved
369 growth of juvenile *Helix aspersa* Müller snails. Lab Anim 40(3): 309–316. doi: 10.1258/002367706777611505.
370 pmid:16803649.
371 Gimbert F, Mench M, Coeurdassier C, Badot PM, de Vaufleury A (2008) Kinetic and dynamic aspects of soil-
372 plant-snail transfer of cadmium in the field. Environ Pollut 152: 736–745. doi: 10.1016/j.envpol.2007.06.044.

373 Gimbert F, de Vaufleury A, Douay F, Scheifler R, Coeurdassier M, Badot PM (2006) Modelling chronic
374 exposure to contaminated soil. A toxicokinetic approach with terrestrial snail *Helix aspersa*. Environ Int 32:
375 866–875. doi: 10.1016/j.envint.2006.05.006.
376 Globisch D, Munzel M, Muller M, Michalakis S, Wagner M et al (2010) Tissue distribution of 5-
377 hydroxymethylcytosine and search for active demethylation intermediates. PloS One 5: e15367.
378 doi: 10.1371/journal.pone.0015367.
379 Gomot A (1997) Dose-dependent effects of cadmium on the growth of snails in toxicity bioassays. Arch Environ
380 Contam Toxicol 33(2): 209–216.
381 Hanna CW, Bloom MS, Robinson WP, Kim D, Parsons PJ et al (2012) DNA methylation changes in whole
382 blood is associated with exposure to the environmental contaminants, mercury, lead, cadmium and bisphenol A,
383 in women undergoing ovarian stimulation for IVF. Hum Reprod 27: 1401–1410. doi: 10.1093/humrep/des038.
384 Head JA, Dolinoy DC, Basu N (2012) Epigenetics for ecotoxicologists. Environ Toxicol Chem 31: 221–227.
385 doi: 10.1002/etc.1707.
386 HHS, US Department of Health and Human Services (2001). Guidance for industry, bioanalytical method
387 validation. http://www. fda. gov/cvm.
388 Hill PW, Amouroux R, Hajkova P (2014) DNA demethylation, Tet proteins and 5-hydroxymethylcytosine in
389 epigenetic reprogramming: an emerging complex story. Genomics 104(5): 324–333.
390 doi: 10.1016/j.ygeno.2014.08.012.
391 Hispard F, Schuler D, de Vaufleury A, Scheifler R, Badot PM, Dallinger R (2008) Metal distribution and
392 metallothionein induction after cadmium exposure in the terrestrial snail *Helix aspersa* (Gastropoda, Pulmonata).
393 Environ Toxicol Chem 27(7):1533–1542. doi: 10.1897/07-232.1.
394 Höckner M, Stefanon K, De Vaufleury A, Monteiro F, Pérez-Rafael S et al (2011) Physiological relevance and
395 contribution to metal balance of specific and non-specific metallothionein isoforms in the garden snail,
396 *Cantareus aspersus*. Biometals 24(6): 1079–1092. doi: 10.1007/s10534-011-9466-x.
397 Hödl E, Felder E, Chabicovsky M, Dallinger R (2010) Cadmium stress stimulates tissue turnover in *Helix*
398 *pomatia*: increasing cell proliferation from metal tolerance to exhaustion in molluscanmidgut gland. Cell Tissue
399 Res 341(1): 159–171. doi: 10.1007/s00441-010-0980-x. pmid:20480182.
400 Hood RD (2016) Developmental and reproductive toxicology: a practical approach. CRC Press, Boca Raton.
401 Hossain MB, Vahter M, Concha G, Broberg K (2012) Low-level environmental cadmium exposure is associated
402 with DNA hypomethylation in Argentinean women. Environ Health Perspect 120: 879–884.
403 doi: 10.1289/ehp.1104600.
404 Itziou A, Kaloyianni M, Dimitriadis V (2011). In vivo and in vitro effects of metals in reactive oxygen species
405 production, protein carbonylation, and DNA damage in land snails *Eobania vermiculata*. Arch Environ Contam
406 Toxicol 60: 697–707. doi: 10.1007/s00244-010-9583-5.
407 Itziou A, Dimitriadis VK (2011) Introduction of the land snail *Eobania vermiculata* as a bioindicator organism
408 of terrestrial pollution using a battery of biomarkers. Sci Total Environ 409(6): 1181–1192.
409 doi: 10.1016/j.scitotenv.2010.12.009.
410 Jiang G, Xu L, Song S, Zhu C, Wu Q, Zhang L, Wu L (2008) Effects of long-term low-dose cadmium exposure
411 on genomic DNA methylation in human embryo lung fibroblast cells. Toxicology 244: 49–55.
412 doi: 10.1016/j.tox.2007.10.028.

413 Jones PA (2012) Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat RevGenet 13:
414 484–492. doi: 10.1038/nrg3230.
415 Kerney MP, Cameron RAD (1979) A field guide to the land snails of Britain and northwestern Europe. William
416 Collins Sons and Co., London.
417 Kucharski R, Maleszka J, Foret S, Maleszka R (2008) Nutritional control of reproductive status in honeybees via
418 DNA methylation. Science 319: 1827–1830. doi: 10.1126/science.1153069.
419 Laskowski R, Hopkin SP (1996) Effect of Zn, Cu, Pb, and Cd on fitness in snails (*Helix aspersa*). Ecotoxicol
420 Environ Saf 34: 59–69. doi:10.1006/eesa.1996.0045.
421 Li W, Liu M (2011) Distribution of 5-hydroxymethylcytosine in different human tissues. J Nucleic Acids. 2011:
422 870726. doi: 10.4061/2011/870726.
423 Lian S, He Y, Li X, Zhao B, Hou R et al (2015) Changes in global DNA methylation intensity and DNMT1
424 transcription during the aging process of scallop *Chlamysfarreri*. J OceanU China 14: 685–690.
425 doi: 10.1007/s11802-015-2507-2.
426 Liu J, Hesson LB, Ward RL (2013) Liquid Chromatography tandem mass spectrometry for the measurement of
427 global DNA methylation and hydroxymethylation. J Proteomics Bioinform S2:005. doi:10.4172/jpb.S2-005
428 Lyko F, Foret S, Kucharski R, Wolf S, Falckenhayn C, Maleszka R (2010) The honey beeepigenomes:
429 differential methylation of brain DNA in queens and workers. PloS Biol 8.11: e1000506. doi:
430 10.1371/journal.pbio.1000506.
431 Moroz LL, Kocot KM, Citarella, MR, Dosung S, Norekian TP et al (2014) The ctenophore genome and the
432 evolutionary origins of neural systems. Nature 510: 110–114. doi:10.1038/nature13400.
433 Moroz LL, Kohn AB (2013) Single-neuron transcriptome and methylome sequencing for epigenomic analysis of
434 aging. Methods Mol. Biol. 1048: 323–352. doi: 10.1007/978-1-62703-556-9_21.
435 Murata A, Baba Y, Ishimoto T, Miyake K, Kosumi K et al. (2015) TET family proteins and 5-
436 hydroxymethylcytosine in esophageal squamous cell carcinoma. Oncotarget 6(27): 23372–23382.
437 doi:10.18632/oncotarget.4281.
438 MWFEP (Ministry of Waters, Forests and Environmental Protection, Romania) (2002) Ordinul nr. 756/1997 al
439 Ministerului Apelor, Padurilor şi Protecţiei Mediului pentru aprobarea Reglementării privind evaluarea poluarii
440 mediului modificat de Ordinul nr. 1144/2002 al Ministerului Apelor si Protectiei Mediului.
441 http://www.unimed.ro/Ordin%20nr.%201144-2002.pdf. Accessed 10.01.2017
442 Nica DV, Popescu C, Draghici GA, Andrica FM, Privistirescu IA, Stöger R (2016) Cadmium hepatotoxicity: an
443 epigenetic gastropod-based approach. SETAC/iEOS Joint Focused Topic, Environmental and (eco)toxicological
444 Omics and Epigenetics: Science, Technology and Regulatory Applications, ISSN 2310-3191.
445 Nica DV, Filimon MN, Bordean DM, Harmanescu M, Draghici GA et al (2015) Impact of soil cadmium on land
446 snails: a two-stage exposure approach under semi-field conditions using bioaccumulative and conchological end-
447 points of exposure. PloS One 10: e0116397. doi: 10.1371/journal.pone.0116397.
448 Notten M, Oosthoek A, Rozema J, Aerts R (2006) The landsnail *Cepaeanemoralis* regulates internal Cd levels
449 when fed on Cd-enriched stinging nettle (*Urticadioica*) leaves at low, field-relevant concentrations. Environ
450 Pollut 139: 296–305. doi: 10.1016/j.envpol.2005.05.007

Oehlmann J, Schulte-Oehlmann U (2003) Molluscs as bioindicators. In: Markert BA, Breure AM, Zechmeister HG (eds) Trace metals and other contaminants in the environment, vol. 6 Elsevier, Oxford, pp. 577–635. doi: 10.1016/S0927-5215(03)80147-9.

Palacios O, Pagani A, Perez-Rafael S, Egg M, Hockner M et al (2011) Shaping mechanisms of metal specificity in a family of metazoan metallothioneins: evolutionary differentiation of molluscmetallothioneins. BMC Biology 9(1): 1. doi: 10.1186/1741-7007-9-4.

Pells S, Koutsouraki E, Morfopoulou S, Valencia-Cadavid S, Tomlinson SR et al (2015) Novel human embryonic stem cell regulators identified by conserved and distinct CpG Island methylation state. PloS one 10(7): e0131102. doi: 10.1371/journal.pone.0131102.

Pierron F, Baillon L, Sow M, Gotreau S, Gonzalez P (2013) Effect of low-dose cadmium exposure on DNA methylation in the endangered European eel. Environ Sci Technol 48: 797–803. doi: 10.1021/es4048347.

Pirola CJ, Scian R, Gianotti TF, Dopazo H, Rohr C et al (2015) Epigenetic modifications in the biology of nonalcoholic fatty liver disease: the role of DNA hydroxymethylation and TET proteins. Medicine 94(36): e1480. doi: 10.1097/MD.0000000000001480.

Rabitsch WB (1996) Metal accumulation in terrestrial pulmonates at a lead/zinc smelter site in Arnoldstein, Austria. Bull Environ Contam Toxicol 56: 734–741.

Rasmussen EM, Vågbø CB, Münch D, Krokan HE, Klungland A et al (2016) DNA base modifications in honey bee and fruit fly genomes suggest an active demethylation machinery with species-and tissue-specific turnover rates. Biochem Biophys Rep 6: 9–15. doi: 10.1016/j.bbrep.2016.02.011.

Riviere G, Wu GC, Fellous A, Goux D, Sourdaine P, Favrel P (2013) DNA methylation is crucial for the early development in the oyster *C. gigas*. Mar Biotechnol 15: 739–753. doi:10.1007/s10126-013-9523-2.

Russell LK, DeHaven JI, Botts RP (1981) Toxic effects of cadmium on the Garden snail (*Helix aspersa*). Bull Environ Contam Toxicol 26: 634–640. doi: 10.1007/BF01622148. pmid:7260433.

Sanders A, Smeester L, Rojas D, De Bussycher T, Wu M et al (2014) Cadmium exposure and the epigenome: Exposure-associated patterns of DNA methylation in leukocytes from mother-baby pairs. Epigenetics 9: 212–221. doi: 10.4161/epi.26798.

Spurgeon DJ, Rowland P, Ainsworth G, Rothery P, Long S, Black HI (2008) Geographical and pedological drivers of distribution and risks to soil fauna of seven metals (Cd, Cu, Cr, Ni, Pb, V and Zn) in British soils. Environ Pollut 153(2): 273–283.

Strepetkaite D, Alzbutas G, Astromskas E, Lagunavicius A, Sabaliauskaite R et al (2016) Analysis of DNA methylation and hydroxymethylation in the genome of crustacean *Daphnia pulex*. Genes 7(1): 1. doi: 10.3390/genes7010001.

Takiguchi M, Achanzar WE, Qu W, Li G, Waalkes MP (2003) Effects of cadmium on DNA-(Cytosine-5) methyltransferase activity and DNA methylation status during cadmium-induced cellular transformation. Exp Cell Res 286: 355–365. doi: 10.1016/S0014-4827(03)00062-4.

Tellez-Plaza M, Wan-yee T, Shang Y, Umans JG, Francesconi KA et al (2014) Association of global DNA methylation and global DNA hydroxymethylation with metals and other exposures in human blood DNA samples. Environ Health Perspect 122(9): 946–954. sdoi: 0.1289/ehp.1306674

Wang H, He L, Song J, Cui W, Zhang Y et al (2016) Cadmium-induced genomic instability in *Arabidopsis*: Molecular toxicological biomarkers for early diagnosis of cadmium stress. Chemosphere 150:258–265. doi: 10.1016/j.chemosphere.2016.02.042.

Wang B, Li Y, Shao C, Tan Y, Cai L (2012) Cadmium and its epigenetic effects. Curr Med Chem 19: 2611–2620. doi: 10.2174/092986712800492913.

Wattanaphansak S, Asawakarn T, Gebhart CJ, Deen J(2008) Development and validation of an enzyme-linked immunosorbent assay for the diagnosis of porcine proliferative enteropathy. J Vet Diagn Invest 20(2): 170–177. doi: 10.1177/104063870802000205.

Wojciechowski M, Rafalski D, Kucharski R, Misztal K, Maleszka J et al (2014) Insights into DNA hydroxymethylation in the honeybee from in-depth analyses of TET dioxygenase. Open Biol 4(8), pii: 140110. doi: 10.1098/rsob.140110.

Zhang J, Mu X, Xu W, Martin FL, Alamdar A, et al (2014) Exposure to arsenic via drinking water induces 5-hydroxymethylcytosine alteration in rat. Sci Total Environ 498:618–625. doi: 10.1016/j.scitotenv.2014.08.009.

Zhang C, Liang Y, Lei L, Zhu G, Chen X et al (2013) Hypermethylations of RASAL1 and KLOTHO is associated with renal dysfunction in a Chinese population environmentally exposed to cadmium. Toxicol Appl Pharmacol 271: 78–85. doi: 10.1016/j.taap.2013.04.025.